Proton NMR Detection of Porphyrins and Cytochrome *c* in Small Unilamellar Vesicles: Role of the Dissociation Kinetic Constant

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ABSTRACT The molecular tumbling of small unilamellar vesicles is not fast enough to enable the detection of ^{1}H NMR signals of molecules associated with phospholipids. We show that relatively fast kinetic exchange of the interacting molecules is able to induce a strong decrease of the residual homonuclear dipolar coupling, allowing the acquisition of sharp signals. At low molecule/lipids molecular ratio, this can be lead to signal broadening due to exchange at intermediate rates on the NMR chemical timescale. However, proton resonances can be easily detected when sufficient lipids are added to prevent the occurrence of any free compounds in solution. This is demonstrated, using lipid signal suppression, in the case of paramagnetic porphyrin derivatives as well as diamagnetic hematoporphyrin. Since several peptides and proteins are expected to be associated with lipids having relatively fast dynamics, this study addresses, as a first example, the interaction of cytochrome *c*.

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Interactions between molecules and membranes are involved in many biological phenomena. Despite the use of membrane models, the description of structural modifications associated with the interaction of molecules with phospholipids remains a major challenge. In this context, recent improvements in NMR spectroscopy have been related to the use of new membrane models such as bicelles or the use of magic-angle spinning (1,2). However, the small unilamellar vesicle (SUV) has long been adopted as the simplest membrane model. Well-resolved lipid signals are observed both by proton and phosphorous NMR. This model has been widely used for analyzing the behavior of phospholipids with interacting molecules. The small size of the vesicles, around 30 nm, induces fast tumbling, which is considered as mainly responsible for the averaging of residual homonuclear dipolar coupling and chemical shift anisotropy. However, the individual movements of each phospholipid are also involved in this averaging. This explains why only a few studies concern the interacting molecules, since the SUV association has long been accepted as causing the broadening of proton NMR beyond detection. Moreover, strong resonances of the protonated phospholipids drastically reduce access to the associated molecule signals, especially when using high ratios of molecules to lipids.

In this letter, we demonstrate that the binding dynamics of the associated molecules is sufficiently fast for the acquisition of good quality proton NMR spectra. The kinetic exchange between the free and bound states has an important impact on proton detection, which accounts for the absence of cholesterol proton signals when this molecule is incorporated in SUVs composed of protonated phospholipids. The very slow kinetic dissociation rate constant, k_{off}, of cholesterol is slow enough to prevent efficient averaging of the residual homonuclear dipolar coupling. Moreover, the vesicular tumbling is not fast enough to detect the proton signals. On the contrary, this explains how the kinetic behavior of the interacting molecules could induce enough additional mobility to allow proton detection. We studied two relevant systems in interaction with SUVs containing protonated phospholipids. The first system corresponds to porphyrin derivatives well known to interact with lipids and which are widely studied because they are involved in photodynamic therapy (3). Based on the results obtained with these compounds and previous data obtained with cytochrome *c* (cyt *c*) in micelles (4), we succeeded in detecting resonances of cyt *c* associated with anionic phospholipids. In paramagnetic molecules, the protons close to the metal, characterizing the active site, give NMR signals well outside the crowded diamagnetic region of 0–10 ppm. In addition, the increase of the paramagnetic proton signal linewidths due to interaction with the lipids is not as dramatic as for diamagnetic compounds. We previously reported a method, called paramagnetic signals enhancement (PASE), for the study of paramagnetic compounds, which offers a major advantage in the case of unlabeled phospholipids (5). Besides the technical observation, this work opens the way of studying the parameters of porphyrin or cyt *c* interactions with various protonated phospholipids. This

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is particularly relevant for cyt *c*, which presents a pretty high versatility of forms depending on the nature of the lipids.

As a first approach, addition of SUV composed of dioleylphosphatidylcholine (DOPC) to a solution of hemin chloride was performed. Because of the insolubility of hemin in acidic media, the addition was performed at pH 10. The solution was then adjusted to pH 2.6 to obtain the pure diaquo-hemin in the high-spin form. Due to the total insolubility of hemin under these conditions, only lipid-associated hemin signals were detected. At a high molar ratio (1:100) of hemin to phospholipids, the low-field shifted resonances of the heme methyl protons can be clearly observed with only a small increase of the linewidth when compared with the micelles of DPC, LysoPC (not shown).

To avoid any problems with insolubility at low pH or the aquo-hydroxo heme ligand exchange or aggregation or some hydrolysis of phospholipids that may occur at the extreme pH used for the study of hemin chloride, we monitored the influence of the heme/SUV ratio on the NMR spectra using an excess of cyanide inducing the formation of *bis*-cyano-hemin, as previously reported for micelles (6). This derivative, existing in a ferric low-spin form, is characterized by heme methyl resonances between 10 and 20 ppm (7). Fig. 1 illustrates the spectra corresponding to various heme/lipids ratios. The spectra of the free hemin derivative and complexes with high lipid concentrations are moderately broad. By contrast, at an intermediate hemin/lipids ratio, very broad resonances are observed with chemical shifts corresponding to fast exchange conditions between the free and bound forms. An increase of the relative phospholipid concentration allows the hemin to become fully associated with lipids, thus preventing the broadening of the resonances caused by hemin exchange between the free and bound forms.

NMR broadening can be induced by various processes such as aggregation or ligand exchange. Definitive interpretation of such behavior requires careful analysis. In our case, further evidence for the positive influence of the kinetic association was obtained from studying the interaction of hematoporphyrin with DOPC SUV. The PASE method cannot be used to detect the diamagnetic proton signals of hematoporphyrin. We used a new protocol sequence, which is described elsewhere, based on a combination of selective and gradient pulses. Fig. 2 shows the portions of spectra corresponding to the meso-protons of hematoporphyrin associated with phospholipids. Fig. 2 *A* presents the spectrum for the meso-protons of hematoporphyrin in the presence of DOPC SUV at a ratio of 1:100, with sharp resonances of ~50 Hz at half-peak height linewidths. At an intermediate hematoporphyrin/lipid ratio, we also observed broad resonances corresponding to chemical exchange at intermediate rate on the chemical shift timescale (titration not shown).

To investigate the influence of the dissociation kinetic constant in more detail, we studied the system with different conditions of temperature, lipid composition, and pH. Previous authors have reported strong pH dependence of the

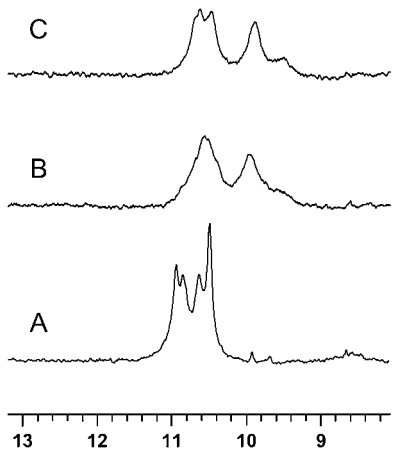

FIGURE 2 Portions of ^{1}H NMR spectra at 323 K corresponding to the meso-protons, using 700 μM molar hematoporphyrin at various porphyrin: DOPC SUV ratios and different pD; intensities are arbitrary. (*A*) fully associated hematoporphyrin, molar ratio 1:100 and pD 7.5; (*B*) partially associated, molar ratio 1:25 at pD 7.5, and (*C*) at pD 10.5.

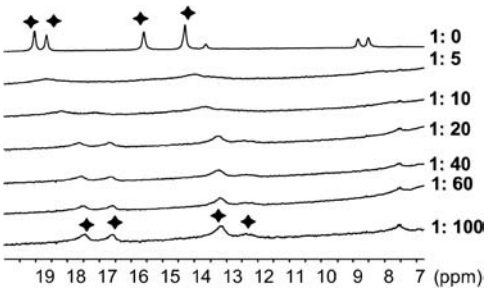

FIGURE 1 Low-field portion of 500-MHz ^{1}H NMR spectra acquired at 313 K at pD 7.5 with the PASE method, using 800 μM of *bis*-cyano-hemin with increasing lipid molar ratio.

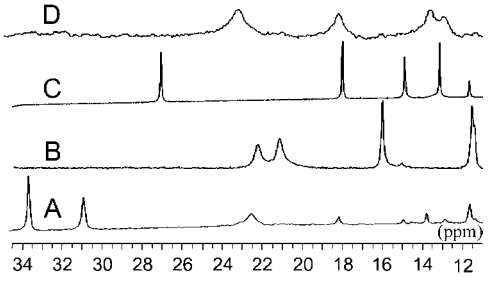

FIGURE 3 Low-field portion of 500 MHz ^{1}H NMR spectra of 500 μM ferri-cyt *c*. (*A*) native; (*B*) cyano adduct; (*C*) cyano adduct with SDS micelles, 1:100; and (*D*) cyano adduct with DOPS/DOPC (2:1) SUV at a molar ratio of 1:150.

k_{off}, with the release of hematoporphyrin from the SUV being governed by neutralization and deprotonation of the carboxylic chains (8,9).

The spectra displayed on Fig. 2, *B* and *C*, correspond to hematoporphyrin interacting with SUV at an intermediate porphyrin/lipid ratio. The difference in the linewidth between the two spectra is not related to the extent of hematoporphyrin binding to the lipids, in agreement with the observed averaged chemical shifts of the meso-protons. Rather, it can be directly correlated with the previously reported variation of the dissociation rate constant, which is much larger under alkaline conditions than at neutral pH. This increase in the kinetic exchange is responsible for reducing the broadening of the averaged resonances.

Based on the results obtained with porphyrin derivatives, we successfully obtained some unprecedented NMR data on cyano-cyt *c* in the presence of SUV composed of a mixture of phospholipids (DOPS/DOPC = 2:1). The portion of the spectrum for the heme methyl protons of cyt *c* is displayed in Fig. 3 *D*. For comparison, Fig. 3 also shows the spectra of cyt *c* forms without lipids. The observed heme methyl resonances are close to the pattern previously observed for SDS micelles (4). In conclusion, we show that it is possible to use proton resonances of molecules or proteins in association with phospholipid vesicles. The transient broadening observed for porphyrin derivatives and cyt *c* is strongly decreased at high protein/lipids ratio, when sufficient lipids are present to prevent the occurrence of any free porphyrin or protein. The narrowing so produced is related to the dissociation rate constant of the molecules interacting with the lipids. This opens up the possibility of proton NMR detection of biomolecules associated with phospholipids, provided the kinetics of the interaction is characterized by a relatively fast k_{off}.

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